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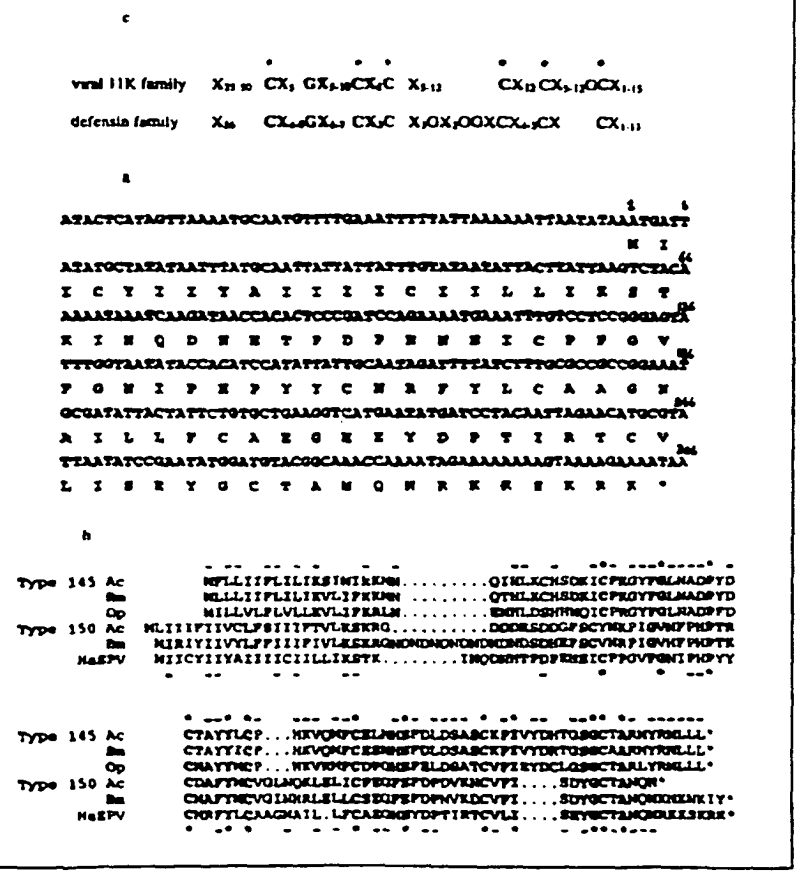
(54) Title: BIOLOGICALLY ACTIVE PROTEINS OF VIRAL ORIGIN

(57) Abstract

A polypeptide from the present invention provides insecticidal polypeptides from insect-infecting viruses, said polypeptides having an hydrophobic N-terminal region of a form characteristic of a signal sequence and a molecular weight of 8-14 kDa. Preferred polypeptides have an amino acid sequence according to the following formula: $X_{25-50}CX_5GX_{5-10}CX_{5-12}GCX_{1-15}$ wherein, in each instance, X represents an amino acid sequence consisting of the particular number of residues indicated. Compositions comprising the polypeptides and transgenic plants expressing the polypeptides are also disclosed.



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BIOLOGICALLY ACTIVE PROTEINS OF VIRAL ORIGIN

This invention relates to member polypeptides of a newly recognised family of polypeptides encoded by insect-infecting viruses and to the uses of their insecticidal properties. Particular applications of the invention reside in methods for producing these polypeptides in cell cultures, the use of the polypeptides in insecticides, and the generation of transgenic plants, particularly crop plants, expressing these polypeptides.

The insect-infecting poxviruses (entomopoxviruses; EPVs) possess molecular, physicochemical and morphological characteristics (12) which support their classification as a sub-family of the *Poxviridae* (11). In addition, EPVs share certain traits with members of unrelated insect virus groups, presumably in response to common requirements for long-term association with those hosts. Thus, for example, EPVs and the nuclear polyhedrosis baculoviruses (NPVs) utilise unrelated proteins to produce functionally equivalent occlusion bodies (spheroids and polyhedra, respectively; 14) which are the agents of horizontal viral transmission (1,13). Conversely, many EPVs (e.g. *Heliothis armigera* EPV; HaEPV), and some NPVs (e.g. *Autographa californica* and *Orgyia pseudotsugata* multi-nucleocapsid NPVs; AcMNPV and OpMNPV, respectively) express a homologous protein which localises in cytoplasmic aggregates sometimes known as spindle bodies (12). The function of these bodies, if any, is not known. The present inventors have previously reported characteristics of the gene encoding the HaEPV spindle body protein (fusolin, 5) and noted that an adjacent open reading frame (ORF) in the HaEPV genome encodes a second example of an homologous gene present in both EPV and baculovirus genomes such as AcMNPV, OpMNPV and *Bombyx mori* singly-enveloped nuclear polyhedrosis virus (BmSNPV) (2, 18, 21).

Analysis of the nucleotide sequence 5' of the intergenic region preceding the HaEPV fusolin gene revealed a short 306 base-pair (bp) ORF (Fig. 1a) potentially encoding a 101 amino acid (aa) polypeptide with a deduced molecular weight of 11482 Da. Nucleotide sequence around the putative translation initiation codon (TAAATG) conforms to that of a consensus late promoter element known to be highly active in both chordopoxvirus and entomopoxvirus contexts (6, 14), suggesting likely expression of the ORF-encoded product.

Comparison of the HaEPV 11K nucleotide and deduced amino acid sequences with other available data (TFastA-based searches on GCG GenBank Release 84; BLAST-based searches of GenBank) showed that the only known related sequences comprised uncharacterised ORFs in the genomes of AcMNPV (ORFs 145 and 150; 2), BmSNPV (ORFs corresponding to nucleotides [nt] 116041-116328 and 120297-120629; 18) and OpMNPV (corresponding to nt 350-637; 21). Analysis of these ORFs, and that from HaEPV, using GCG algorithms showed that they comprise two distinct but related homology groups, which, by reference to AcMNPV nomenclature (2), we denote as Type 145 and 150 groups. Pairwise comparisons (GCG Gap algorithm at default settings) showed that the HaEPV 11K protein is a member of the Type 150 group (Table 1), and, broadly, found 42-74% identity between deduced amino acid sequences in that group. Comparisons of members of the Type 145 group showed identities of 66-90% within that group; in contrast, comparison of sequences from the two different groups showed identities of 27-36% (Table 1). Alignment of all six sequences with the GCG PileUp algorithm (default settings) showed conservation of five cysteine residues distributed throughout the molecules, and of a GC(A/T)A motif near the C-terminal end (Figure 1b). In addition, the positions of a sixth cysteine residue and a glycine residue present in all six sequences (HaEPV 11K C^{38} and G^{44}) were very similar. Regions of amino acids between the conserved cysteines were variable in both residue number and sequence, and in combination the aligned molecules could thus be assigned the descriptor $X_{31-44}CX_5GX_{7-8}CX_5CX_{7-10}CX_{12}CX_{8-10}GCX_{5-12}$. Further analysis of deduced amino acid sequences (GCG PepPlot) from members of the two groups showed similar hydrophobicity profiles for each, including the presence of regions of very high hydrophobicity, compatible with membrane association or secretion, at the N-terminus of each.

Table 1: Relationships (% identities of deduced amino acid sequences) between 11K homologues as determined by pairwise comparisons with the GCG Gap algorithm at default settings.

		Type 145 ORFs			Type 150 ORFs	
		AcMNPV	BmSNPV	OpMNPV	AcMNPV	BmSNPV
Type 145	BmSNPV	90				
	OpMNPV	66	69			
Type 150	AcMNPV	32	29	33		
	BmSNPV	29	27	33	74	
	HaEPV	36	30	31	42	43

Given these observations it was of interest to determine whether any or all of these ORFs were expressed during the course of viral infection. As shown hereinafter (Figure 2), evidence was obtained indicating that both the HaEPV and AcMNPV Type 150 ORFs are indeed expressed and that this occurs at different levels in the different viruses. While the function(s) of each of these proteins under natural conditions of expression remains to be determined, we have shown that expression of the HaEPV protein in an heterologous system (ie, an infectious recombinant baculovirus; Figure 3) and over-expression of AcMNPV ORF 150 in the same system in both sense and anti-sense configurations confers significantly enhanced insecticidal activity (Table 2 and Figure 4).

Thus, in a first aspect, the present invention provides a polypeptide from an insect-infecting virus, said polypeptide having an hydrophobic N-terminal region of a form characteristic of a signal sequence and a molecular weight of 8-14 kDa, in a substantially pure form.

Preferably, the polypeptide has a molecular weight in the range of 9-13 kDa, more preferably, 10-12 kDa.

The characteristic form of signal sequences is as set out in Gierasch, L. M. Biochemistry 28, 923-930 (1989), the entire disclosure of which is incorporated herein by reference.

In a second aspect, the present invention provides a viral polypeptide having an amino acid sequence of the formula:

$X_{25-50}CX_5GX_{5-10}CX_5CX_{5-12}CX_{12}CX_{5-12}GCX_{1-15}$

wherein, in each instance, X represents an amino acid sequence consisting of the particular number of residues indicated, the viral polypeptide being in a substantially pure form.

In a third aspect, the present invention provides a viral polypeptide having an amino acid sequence of the formula:

$X_{31-44}CX_5GX_{7-8}CX_5CX_{7-10}CX_{12}CX_{6-10}GCX_{5-12}$

wherein, in each instance, X represents an amino acid sequence consisting of the particular number of residues indicated, the viral polypeptide being in a substantially pure form.

In a fourth aspect, the present invention provides a viral polypeptide having an amino acid sequence/corresponding to the conserved ORF Type 150 of the formula:

$X_{1-2}IX_3I_2X_5IXIX_4KSX_{8-18}DX_{4-5}CX_5GX_{2-3}PHPX_2CX_2FYXCX_3NX_2LX_{0-1}$
LXCXEGXEXDPX$_4$CVXISXYGCTANQNX$_{0-7}$

wherein, in each instance, X represents an amino acid sequence consisting of the particular number of residues indicated, the viral polypeptide being in a substantially pure form.

In a fifth aspect, the present invention provides a viral polypeptide having an amino acid sequence/corresponding to the conserved ORF Type 145 formula:

$X_2LLX_2FLXLXKX_4KX_2NX_2HLX_2HX_3ICPXGYFGLNADPXDCXAYYXCPHKVX$
$MFCX_3HEFXLDSAXCXPIXYDX_2GSGCXARXYRNLLL$

wherein, in each instance, X represents an amino acid sequence consisting of the particular number of residues indicated, the viral polypeptide being in a substantially pure form.

In a sixth aspect, the present invention provides a viral polypeptide including an amino acid sequence corresponding to a portion of the ORF Type 145 formula characterised thus:

$X_4HLX_2HX_3ICPXGYFGLNADPXDCXAYYXCPHKVXMFCX_3HEFXLDSAXCXP$
$IXYDX_2GSGCXARXYRNLLL$

wherein, in each instance, X represents an amino acid sequence consisting of the particular number of residues indicated, the viral polypeptide being in a substantially pure form.

The sequences represented by X in the second to sixth aspects consist of one or more α-amino acids selected from A, R, N, D, C, Q, E, G, H,

I, L, K, M, F, P, S, T, W, Y, V (standard one letter codes) and corresponding Nα-alkalamino acids.

Preferably, the viral polypeptides of the first to sixth aspects are from insect poxviruses (e.g. entomopoxviruses) or baculoviruses (e.g. nuclear polyhedrosis viruses). Further, they preferably exhibit insecticidal activity.

In a seventh aspect, the present invention provides a polypeptide having an amino acid sequence substantially corresponding to that shown in Figure 1a or showing ≥ 25%, more preferably ≥ 40%, identity to that shown in Figure 1a, in substantially pure form.

Preferably, the polypeptide of the first to seventh aspects has an amino acid sequence substantially corresponding to any one of the HaEPV, AcMNPV, BmSNPV or OpMNPV sequences shown in Figure 1b.

The term "substantially corresponding" as used herein in relation to amino acid sequences is intended to encompass minor variations in the amino acid sequence which do not result in a decrease in biological activity of the polypeptide. These variations may include conservative amino acid substitutions. Amino acid grouping for the substitutions envisaged are:- G,A,V,L,M; D,E; N,Q; S,T; K,R,H; F,Y,W,H; and P,Nα-alkalamino acids.

In an eighth aspect, the present invention provides a polypeptide which binds to a monospecific anti-HaEPV 11K polypeptide antibody.

The polypeptide of any one of the first to eighth aspects may be purified from a natural source, for example, host cells or insects infected with natural strains of HaEPV, AcMNPV, BmSNPV or OpMNPV. However, in order to produce the polypeptide in commercial quantities, it is preferred to produce a cell culture containing an expression construct or vector including a DNA sequence encoding the polypeptide. For example, a DNA molecule including the DNA sequence shown at Figure 1a from nucleotide 1 to 306 or recorded in Genbank under the Accession No. M59422 locus NPAIEN (AcMNPV), L33180 locus NPHT3COMP (BmSNPV), or M62414 locus NPOIE1A (OpMNPV), may be introduced into a suitable expression vector and used to transform or infect a cell culture of, for example, *E. coli*, *Spodoptera frugiperda* Sf9, *Heliothis zea* Hz-BCIRL-AM1 (USDA, Biological Control of Insects Research Laboratory, Columbia, Missouri), yeast, or Chinese Hamster Ovary (CHO) cells.

The invention therefore also relates to methods for the production of polypeptides involving expression of a DNA molecule encoding a polypeptide according to any one of the first to eighth aspects.

It should also be understood that it may be possible to obtain, and produce, fragments of the polypeptide according to the invention, which exhibit biological activity such as insecticidal activity. Such fragments and methods of producing same are also to be regarded as forming part of the present invention.

The polypeptides according to the invention, or biologically active fragments thereof, show promise for control of pest insect species, for example, noctuid species. Thus, the invention also extends to methods for controlling the proliferation of pest insects, comprising applying to an infested area one or more polypeptides, and/or one or more or insecticidally-active fragments thereof, according to any one of the first to eighth aspects, optionally in admixture with an agriculturally-acceptable carrier.

Insecticide compositions comprising one or more polypeptides, and/or insecticidally-active fragments thereof, according to the invention are also to be regarded as forming part of the invention.

DNA molecules encoding the polypeptides of the invention could also be introduced into insecticidal recombinant viruses, in a manner which achieves expression from their native promoter, or a heterologous promoter, and improves the toxicity of such viruses for pest insect species. Alternatively, such DNA molecules could be introduced into crop plants, such that the crop plants produce insecticidal amounts of the polypeptide according to the invention. Methods for introducing and achieving expression of heterologous (e.g. toxin) genes in plants are described in Australian patent specification No. 51630/90 and United States Patent specification No. 5380831. The entire disclosure of both of these specifications is incorporated herein by reference.

The invention will now be further described with reference to the following non-limiting examples and accompanying figures.

Brief description of the figures.

Figure 1: (a) Nucleotide and deduced amino acid sequences of the HaEPV 11K protein. (b) Alignment of deduced amino acid sequences of 11K

homologues from HaEPV, AcMNPV (Ac), BmSNPV (Bm) and OpMNPV (Op) as determined by the GCG PileUp algorithm at default settings. Lower case "k" at residue 17 of AcMNPV Type 145 ORF indicates a lysine residue whose existence is deduced by comparison of the AcMNPV sequence with those of BmSNPV and OpMNPV, and whose coding requires omission of a single nucleotide (nt) residue (#126292) from the sequence described by Ayres *et al.* (2). Dashes above and below aligned sequences indicate residues which are conserved in Type 145 and 150 ORFs, respectively; asterisks indicate residues conserved in all members of the polypeptide family. (c) Relative positions of conserved cysteine and glycine in viral 11K proteins and insect defensins. Bold type shows regions of similarity at a position corresponding to the N-terminus of mature defensin proteins.

Figure 2: (a, b) RT-PCR detection of 11K protein-encoding RNA in (a) AcMNPV- and (b) HaEPV-infected Sf9 cells. Lanes 1-6 and 7-12 show products from reactions (or for HaEPV, reamplifications of reactions) which used 1 μg of total RNA from mock- and virus-infected cells, respectively, as template. Reactions shown in lanes 1 & 2 and 7 & 8 used template RNA isolated 48 hr post-infection (pi), and were initiated with either 4U of AMV-RT and 100U of MMLV-RT (1 & 7), or 25U of RNaseA (2 & 8). Lanes 3 & 4 and 9 & 10, and 5 & 6 and 11 & 12, used template RNA isolated 96 and 120 hr pi, respectively, treated in a manner identical to that described above. Reactions in lane 13 contained AMV- and MMLV-RTS and appropriate primers, without added template. Marker (M) is 100 bp ladder, numbers at left show size (bp). (c) Northern blot detection of AcMNPV 11K-related transcripts. Total RNA was isolated from mock- and AcMNPV-infected cells at indicated times pi (hr); 5 μg of each sample were hybridised to a full-length AcMNPV 150 ORF-specific probe. Lengths of RNA markers (kb) are shown at left.

Figure 3: Production of HaEPV 11K protein by a baculovirus expression vector. *S. frugiperda* (Sf9) cells infected with parental virus (BacPac6 AcMNPV, lanes 1 & 3) or an HaEPV 11K-expressing recombinant (11K:AcMNPV, lanes 2 & 4) were harvested at 96 hr pi, and constituent proteins analysed on 15% Tris-tricine SDS-PAGE. Coomassie staining of the separated proteins (lanes 1 & 2) showed high level β-galactosidase

production by the parental virus (lane 1, arrow), but neither this protein, nor a novel product, could be visualised in separated constituents of 11K:AcMNPV-infected cells (lane 2). Nevertheless, Western blotting of samples with a monospecific anti-HaEPV 11K antibody (lanes 3 & 4) demonstrated the presence of an antigenically-related protein unique to extracts of 11K:AcMNPV-infected cells (lane 4). Sizes (kDa) of M_r marker proteins (M) are indicated.

Figure 4: (a) Neonate *Chrysodeixis argentifera* (Lepidoptera: Noctuidae) larvae were fed preoccluded recombinant AcMNPV virions at doses sufficient to cause 100% mortality 10 days post-ingestion. The parental recombinant (BacPac6 AcMNPV; dashed line) expressed the β-galactosidase protein (see Figure 3), and was used as a control. The "experimental" recombinant (11K:AcMNPV; solid line) expressed the HaEPV 11K protein (Figure 3). The time at which 50% of the larvae fed the former had died (LT_{50}: β-gal) was estimated at 6.4 days, while for those fed the latter it (LT_{50}: E11K) was 4.8 days. Expression of the HaEPV 11K protein was thus responsible for a reduction of 25% in the LT_{50} of the latter virus. (b) In a second experiment (using methods described in [a] above) *C. argentifera* larvae were fed the recombinant viruses at doses sufficient to cause 70% mortality 10 days post-ingestion. In this experiment the LT_{50}:β-gal was estimated at 7.2 days, and the LT_{50}:E11K at 4.7 days. Thus at this dose rate expression of the HaEPV 11K protein was responsible for a reduction of 35% in viral LT_{50}.

Example 1

RT-PCR was used to detect RNA of appropriate sequence in preparations of *Spodoptera* Sf9 cells (American Type Culture Collection) infected with either HaEPV or AcMNPV (each at a multiplicity of 2), and harvested at 48, 96 or 120 hr post-infection (pi). Total RNA was isolated from mock - and virus-infected Sf9 cells (4); 10-15 µg of total cellular RNA was heated at 95°C for 5 min, cooled to 60°C prior to addition of 7 U of Rnase-free DNase, incubated at 37°C for 30 min, then phenol extracted and precipitated. cDNA synthesis reactions (25 µl) contained 1 µg of prepared RNA as template, 8 U of avian myoblastosis virus and 100 U of Maloneys

murine leukaemia virus reverse transcriptases (RTs). 1 mM dNTPs and 4μM oligonucleotide (final concentrations), and were incubated for 90 min at 37°C. Mock cDNA synthesis reactions contained 25 U of RNase instead of RTs; in each case 10 μl of the synthesis reaction was used as template for PCR amplification.

All PCR amplifications used 4-6 U of *Taq* DNA polymerase in 100 μl reaction volumes containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris, pH 8.3 and 0.1% gelatin (final concentrations). A 5 min pre-incubation at 80°C was included prior to addition of the polymerase, then the reaction proceeded through 25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min.

cDNA synthesis reactions were primed with oligonucleotides (oligos) EPP11C or NPP11C (5'CATGGATCCAATTTATAATTATTTTCTTTTAC and TTAGTTTTGGTTAGCGGTACATCC, respectively), then amplified after addition of oligos EPP11N or NPP11N1 (5'CGCGGATCCAACATGATTATATGCTATATAATTT and TTAGGATCCAACATGTTAATTATAATATTC, respectively).

As shown in Figure 2, bands of an expected size (about 300 bp) were amplified under experimental, but not control conditions, and their identities were verified by subsequent cloning and sequencing. While the AcMNPV ORF 150 product could be easily detected in a single round of RT-PCR, that of HaEPV required a further round of PCR, using 10 μl of the original 100 μl RT-PCR as template, before detection was possible. Control preparations were treated in an identical manner, and remained devoid of detectable product (Fig. 2b).

Results from Northern blotting protocols supported those derived by RT-PCR. We used random-labelled probes synthesised on PCR-generated templates, with stringent hybridisation conditions (65°C), and total RNA preparations made 12, 24, 48, 72 or 96 hr after viral or mock infection. As shown (Fig. 2c), a probe specific for AcMNPV ORF 150 generated with oligos NPP11DN (5'CAATAAATAAGTTAACACCCAACATTGATCATTATAATATTC) and NPP11C detected a discrete 11K-related transcript about 450 bases in length in 5 μg RNA samples from AcMNPV-infected Sf9 cells harvested 48, 72 or 96 hr pi, but not in parallel preparations from uninfected cells. We were, however, not able to detect 11K-related transcripts in RNA preparations from

HaEPV-infected Sf9 or *Helicoverpa zea* (e.g. BCIRL-Hz-AM1; 19)-derived cells by this method.

Together, these data indicate that under the *in vitro* conditions used, transcription of the HaEPV gene was very limited, despite the presence of the TAAATG poxvirus late promoter motif.

AcMNPV ORF150 has not previously been recognised as a functional gene; in fact, initial sequence- and context-based assessment of characteristics of the ORF, based on assumption of translation initiation at the first in-frame deduced methionine (M) residue, suggested that it was unlikely to be so (2). Our examination of coding sequence of the ORF, together with comparison of the homologous deduced amino acid sequences, suggested that translation was more likely to initiate at a second ATG triplet located 15 residues "downstream" of the first, as shown in Fig. 1b.

On the basis of results presented above it is concluded that both the HaEPV 11K ORF and the AcMNPV ORF150 are functional genes, and it is suggested that their products are part of a previously unrecognised family of polypeptides common to EPVs and NPVs. The relationship between member ORFs of the two types described here has not previously been recognised. Likewise, ORF145 of AcMNPV has not previously been recognised in the form presented here; as described in the legend to Figure 1b, omission of a single nt from the published AcMNPV sequence (2, L22858) alters the predicted starting position of ORF145 to nt 126244, and thus adds 18 amino acids whose sequence and predicted peptide leader function are highly conserved in homologues from BmSNPV and OpMNPV. AcMNPV sequence as published (2) predicts the start of ORF145 as being at nt 126299, a position corresponding to the aa residue shown as M19 in Figure 1b. That sequence can still be aligned with those shown in Figure 1b, and then simply lacks the predicted N-terminal sequence shown there.

A number of families of biologically active peptides containing six conserved cysteine residues, and a more variable number of conserved glycines, have been described. These include the anti-microbial defensins encoded by insects and mammals (10, 16), the leech antihemostatic proteins (15), and various neurologically-active peptides such as the cono- and charybdotoxins of molluscs and scorpions (20, 3). A conotoxin has previously been described from AcMNPV (9), and a conotoxin-like protein of the *Campoletis sonorensis* polydnavirus (8) has been implicated in

suppression of the host immune response to parasitoid eggs (17). Comparison of the 11K viral peptide descriptor with those of these and other peptide families, using the first conserved cysteine as a reference point, revealed a similarity with the N-terminal region of the insect defensins (Fig. 1c), but no other notable relationships.

Peptides of the type described above are generally translated in pre- or pre/pro-forms, and attain mature size and configuration through secretion and other processing events. The hydrophobic signal-like sequences deduced as present in all six viral products suggested the possibility of a similar mode of processing for these proteins. In an attempt to identify the translation product of the HaEPV 11K gene we prepared polyclonal rabbit antiserum to a bacterial fusion protein comprising an N-terminal glutathione-S-transferase sequence (from pGex2T; Pharmacia Biotech) and a C-terminal 11K gene element. After failure to produce a fusion protein containing the entire 11K gene sequence, we cloned a fragment whose 5' terminus was generated by SspI digestion between coding nucleotides 47 and 48. This construct thus lacked the 16 (mostly hydrophobic) N-terminal amino acids of the full length 11K protein: transformed bacteria produced a novel polypeptide of expected size following induction of expression by IPTG. This band was excised from polyacrylamide gels, re-run on agarose gels, and the resultant band excised, solubilised and used for immunisation.

In a parallel attempt to produce the HaEPV 11K protein in quantities sufficient to allow investigation of putative post-translational processing and other biological properties, a recombinant AcMNPV was made containing the 11K-encoding gene under control of the polyhedrin promoter. We placed a PCR-generated (using the primers EPP11N/C described above) full-length copy of the gene in transfer vector pVL941 (PharMingen), verified the construct by sequencing, then, following co-transfection of AcMNPV DNA (BacPac6; Clontech) and construct DNA into Sf9 cells, recovered and purified a putative AcMNPV recombinant (11K:AcMNPV). The recombinant status of this isolate was subsequently verified by demonstration of absence of expression of the β-galactosidase marker protein (Figure 3, lanes 1 & 2).

The monospecific anti-HaEPV 11K serum recognised a protein with a relative molecular mass of about 15K in lysates of cells harvested 63 or 96 hr after infection with the recombinant 11K:AcMNPV (e.g. Fig. 3, lane 4), even though no corresponding protein could be visualised on Coomassie-stained

polyacrylamide gels containing those samples (Fig. 3, lane 2). The same serum did not, however, detect antigen in lysates of AcMNPV (parental BacPac6)-infected cells (Fig. 3, lane 3) or mock-infected cells (not shown) prepared and analysed in parallel, or in preparations of HaEPV-infected *Helicoverpa zea* cells harvested 2, 4, 6 or 10 days pi. This latter result is again consistent with the low levels of HaEPV 11K gene expression described above.

Antigen was not detected in medium (Grace's with 10% foetal calf serum) collected from cultures of 11K:AcMNPV-infected Sf9 cells (collected 72 hr pi) tested in either an unprocessed form, or after concentration (by centrifugation through Centricon 30K filters followed by acetone precipitation). We were thus unable to link the low observed abundance of the HaEPV 11K protein in baculovirus expression vector-infected cells with a post-translational or secretion pathway, although existence of the same remains an attractive hypothesis.

Example 2

Neonate *Chrysodeixis argentifera* (Lepidoptera: Noctuidae) larvae were fed preoccluded recombinant AcMNPV virions at doses sufficient to cause 100% mortality 10 days post-ingestion. The parental recombinant (BacPac6 AcMNPV; dashed line) expressed the β-galactosidase protein (see Figure 3), and was used as a control. The "experimental" recombinant (11K:AcMNPV; solid line) expressed the HaEPV 11K protein (Figure 3). The time at which 50% of the larvae fed the former had died (LT_{50}: β-gal) was estimated at 6.4 days, while for those fed the latter it (LT_{50}: E11K) was 4.8 days. Expression of the HaEPV 11K protein was thus responsible for a reduction of 25% in the LT_{50} of the latter virus.

In a second experiment (using methods described in [a] above) *C. argentifera* larvae were fed the recombinant viruses at doses sufficient to cause 70% mortality 10 days post-ingestion. In this experiment the LT_{50}:β-gal was estimated at 7.2 days, and the LT_{50}:E11K at 4.7 days. Thus at this dose rate expression of the HaEPV 11K protein was responsible for a reduction of 35% in viral LT_{50}.

A third series of experiments used four different recombinant AcMNPV isolates, containing either a β-galactosidase gene (BacPac6

AcMNPV), the HaEPV 11K gene (11K:AcMNPV), and the AcMNPV ORF150 gene in either sense (+Ac150:AcMNPV) or anti-sense (-Ac150:AcMNPV) orientations, respectively (Table 2). The biological activity of preparation of each recombinant was assessed by determining the quantity required to kill 50% of test larvae (neonate *C. argentifera*) within 7 days of ingestion ($LD_{50:7}$). Neonate *C. argentifera* larvae were then fed a quantity of each recombinant virus ten-fold greater than the assessed $LD_{50:7}$, and the time required for that dose to kill 50% of exposed larvae (LT50) was determined. As shown in Table 2, recombinants which over-expressed either the HaEPV 11K protein or the AcMNPV ORF 150 homologue had LT_{50}s significantly shorter than that of the parental recombinant, and hence possessed enhanced insecticidal activity. Over-expression of AcMNPV ORF150 in an anti-sense configuration - a strategy widely used to "knock out" normal gene function-also resulted in a reduction in LT_{50} of recombinant virus of that form, suggesting that this is an alternative method for deployment of these nucleotide sequences.

Table 2

	LT_{50}*	s.e.	n
BacPac 6 AcMNPV	6.07	0.11	4
11K:AcMNPV	4.86	0.17	4
+Ac150:AcMNPV	5.09	-	2
-Ac150:AcMNPV	4.62	-	2

* data show mean LT_{50} values (days) with standard error of mean, based on 'n' determinations.

References

1. ADAMS, J.R., and McCLINTOCK, J.T., In "Atlas of Invertebrate Viruses" (J.R. Adams and J.R. Bonami Eds.) pp. 87-204. CRC Pres, Boca Raton, 1991.

2. AYRES, M.D., HOWARD, S.C., KUZIO, J., LOPEZ-FERBER, M., and POSSEE, R.D., Virology 202, 585-605 (1994).

3. BONTEMS, F., ROUMESTAND, C., GILQUIN, B., MENEZ, A., and TOMA, F., Science 254. 1521-1523 (1991).

4. CHOMCZYNSKI, P., and SACCHI, N., Anal. Biochem. 162, 156-159 (1987).

5. DALL, D.J., SRISKANTHA, A., VERA, A.P., LAI-FOOK, J., and SYMONDS, T.M., J. gen. Virol. 74, 1811-1818 (1993).

6. DAVISON, A.J. and MOSS, B., J. Mol. Biol. 210, 771-784 (1989).

7. DEVEREUX, J., HAEBBERLI, P., and SMITHIES, O., Nucl. Acids Res. 12, 387-395 (1984).

8. DIB-HAJI, S.D., WEBB, B.A., and SUMMERS, M.D., Proc. Natl. Acad. Sci. USA. 90, 3765-3769 (1993).

9. ELDRIDGE, R., LI, Y., and MILLER, L.K., J. Virol. 66, 6563-6571 (1992).

10. FAYE, I., and HULTMARK, D., In "Parasites and Pathogenes of Insects" (Beckage, N.E., Thompson, S.N. and Federici, B.A., Eds.) Vol. 2; pp. 25-53. Academic Press, New York, 1993.

11. FRANCKI, R.I.B., FAUQUET, C.M., KNUDSON, D.L., and BROWN, F. (Eds.), "Classificaation and nomenclature of viruses." Springer-Verlag, New York, 1991.

12. GOODWIN, R.H., MILNER, R.J., and BEATON, S.D., In "Atlas of Invertebrate Viruses" (Adams, J.R., and Bonami, J.R., Eds.) pp. 393-434. CRC Press, Boca Raton, 1991.

13. GRANADOS, R.R., In "Pathogenesis of Invertebrate Microbial Dieases" (Davidson, E.W., Ed.) pp. 101-126. Allenheld Osmun, Totowa, N.J., 1981.

14. HALL, R.L., and MOYER, R.W., J. Virol. 65, 6516-6527 (1991).

15. KREZEL, A.M., WAGNER, G., SEYMOUR-ULMER, J., and LAZARUS, R.A., Science 264, 1944-1947 (1994).

16. LEGRER, R.I., LICHTENSTEIN, A.K., and GANZ, T., Annu. Rev. Immunol. 11, 105-128 (1993).

17. LI, X., and WEBB, B.A., J. Virol. 68, 7482-7489 (1994).

18. MAEDA, S. unpublished. "*Bombyx mori* nuclear polyhedrosis virus (individual isolate T3) DNA, complete genome". GenBank accession number L33180.

19. McINTOSH, A.H., and IGNOFFO, C.M., J. Invertebr. Pathol. 37, 258-264 (1981).

20. OLIVERA, B.M., RIVIER, J., SCOTT, J.K., HILLYARD, D.R., and CRUZ, L.J., J. Biol. Chem. 266, 22067-22070 (1991).

21. THEILMANN, D.A., and STEWART, S. (1991). Identification and characterisation of the IE1 gene of *Orgvia pseudotsugata* multicapsid nuclear polyhedrosis virus. Virology 180, 492-508.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims:

1. A polypeptide from an insect-infecting virus, said polypeptide having an hydrophobic N-terminal region of a form characteristic of a signal sequence and a molecular weight of 8-14 kDa, in a substantially pure form.

2. A polypeptide according to claim 1, wherein said polypeptide has a molecular weight of 9-13 kDa.

3. A polypeptide according to claim 1, wherein said polypeptide has a molecular weight of 10-12 kDa.

4. A viral polypeptide having an amino acid sequence of the formula:

$X_{25-50}CX_5GX_{5-10}CX_5CX_{5-12}CX_{12}CX_{5-12}GCX_{1-15}$

wherein, in each instance, X represents an amino acid sequence consisting of the particular number of residues indicated, the viral polypeptide being in a substantially pure form.

5. A viral polypeptide having an amino acid sequence of the formula:

$X_{31-44}CX_5GX_{7-8}CX_5CX_{7-10}CX_{12}CX_{8-10}GCX_{5-12}$

wherein, in each instance, X represents an amino acid sequence consisting of the particular number of residues indicated, the viral polypeptide being in a substantially pure form.

6. A viral polypeptide having an amino acid sequence/corresponding to the conserved ORF Type 150 of the formula:

$X_{1-2}IX_3I_2X_5IXIX_4KSX_{8-18}DX_{4-5}CX_5GX_{2-3}PHPX_2CX_2FYXCX_3NX_2LX_{0-1}$
$LXCXEGXEXDPX_4CVXISXYGCTANQNX_{0-7}$

wherein, in each instance, X represents an amino acid sequence consisting of the particular number of residues indicated, the viral polypeptide being in a substantially pure form.

7. A viral polypeptide having an amino acid sequence/corresponding to the conserved ORF Type 145 formula:

$X_2LLX_2FLXLXKX_4KX_2NX_2HLX_2HX_3ICPXGYFGLNADPXDCXAYYX$
$CPHKVXMFCX_3HEFXLDSAXCXPIXYDX_2GSGCXARXYRNLLL$

wherein, in each instance, X represents an amino acid sequence consisting of the particular number of residues indicated, the viral polypeptide being in a substantially pure form.

8. A viral polypeptide including an amino acid sequence/corresponding to a portion of the ORF Type 145 formula characterised thus:

$X_4HLX_2HX_3ICPXGYFGLNADPXDCXAYYXCPHKVXMFCX_3HEFXL$
$DSAXCXPIXYDX_2GSGCXARXYRNLLL$

wherein, in each instance, X represents an amino acid sequence consisting of the particular number of residues indicated, the viral polypeptide being in a substantially pure form.

9. A viral polypeptide according to any one of the preceding claims, wherein said polypeptide is from insect poxviruses or baculoviruses.

10. A viral polypeptide according to claim 9, wherein said polypeptide is from an entomopoxvirus.

11. A viral polypeptide according to claim 10, wherein said polypeptide is from HaEPV.

12. A viral polypeptide according to claim 9, wherein said polypeptide is from a nuclear polyhedrosis virus.

13. A viral polypeptide according to claim 12, wherein said polypeptide is from AcMNPV, OpMNPV or BmSNPV.

14. A viral polypeptide according any one of the preceding claims, wherein said polypeptide exhibits insecticidal activity.

15. A polypeptide having an amino acid sequence showing ≥ 25% identity to that shown in Figure 1a.

16. A polypeptide having an amino acid sequence showing ≥ 40% identity to that shown in Figure 1a.

17. A polypeptide having an amino acid sequence substantially corresponding to that shown in Figure 1a.

18. A polypeptide having an amino acid sequence substantially corresponding to any one of the HaEPV, AcMNPV, BmSNPV or OpMNPV sequences shown in Figure 1b.

19. A polypeptide which binds to a monospecific anti-HaEPV 11K polypeptide antibody.

20. A biologically-active fragment of a polypeptide according to any one of the preceding claims.

21. A method of production of a polypeptide according to any one of claims 1 to 19 or an insecticidally-active fragment thereof, comprising expression from a host cell harbouring a DNA molecule encoding said polypeptide or fragment.

22. A method according to claim 21 wherein said host cell is an isolated insect cell line.

23. A method according to claim 22, wherein said host cell is selected from Sf9 cells and Hz-BCIRL-AM1 cells.

24. A method for controlling the proliferation of pest insects, comprising applying to an infested area one or more polypeptides according to any one of claims 1 to 19 and/or one or more insecticidally-active fragments thereof, optionally in admixture with an agriculturally-acceptable carrier.

25. An insecticide composition comprising one or more polypeptides according to any one of claims 1 to 19 and/or one or more insecticidally-active fragments thereof in admixture with a suitable carrier.

26. An expression vector including a nucleotide sequence encoding a polypeptide according to any one of claims 1 to 19 or an insecticidally-active fragment thereof.

27. A transgenic plant which expresses insecticidal amounts of a polypeptide according to any one of claims 1 to 19 or an insecticidally-active fragment thereof.

28. A transgenic plant according to claim 27, said plant being selected from crop plants.

Figure 1a

```
                                                                  1     6
ATACTCATAGTTAAAATGCAATGTTTTGAAATTTTTATTAAAAAATTAATATAAATGATT
                                                                  M   I
                                                                       66
ATATGCTATATAATTTATGCAATTATTATTATTTGTATAATATTACTTATTAAGTCTACA
I  C  Y  I  I  Y  A  I  I  I  I  C  I  I  L  L  I  K  S  T
                                                                      126
AAAATAAATCAAGATAACCACACTCCCGATCCAGAAAATGAAATTTGTCCTCCGGGAGTA
K  I  N  Q  D  N  H  T  P  D  P  E  N  E  I  C  P  P  G  V
                                                                      186
TTTGGTAATATACCACATCCATATTATTGCAATAGATTTTATCTTTGCGCCGCCGGAAAT
F  G  N  I  P  H  P  Y  Y  C  N  R  F  Y  L  C  A  A  G  N
                                                                      246
GCGATATTACTATTCTGTGCTGAAGGTCATGAATATGATCCTACAATTAGAACATGCGTA
A  I  L  L  F  C  A  E  G  H  E  Y  D  P  T  I  R  T  C  V
                                                                      306
TTAATATCCGAATATGGATGTACGGCAAACCAAAATAGAAAAAAAAGTAAAAGAAAATAA
L  I  S  E  Y  G  C  T  A  N  Q  N  R  K  K  S  K  R  K  *
```

Figure 1(b)

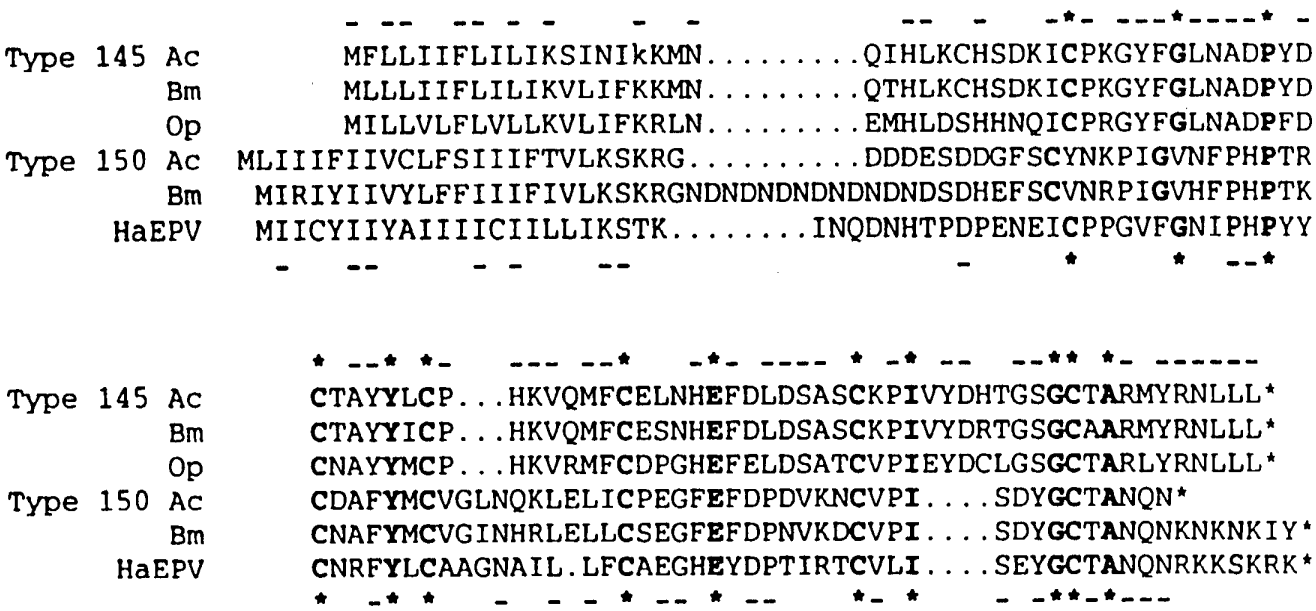

Type 145 Ac MFLLIIFLILIKSINIkKMN.....QIHLKCHSDKICPKGYFGLNADPYD
Bm MLLLIIFLILIKVLIFKKMN.....QTHLKCHSDKICPKGYFGLNADPYD
Op MILLVLFLVLLKVLIFKRLN.....EMHLDSHHNQICPRGYFGLNADPFD
Type 150 Ac MLIIIFIIVCLFSIIIFTVLKSKRG.....DDDESDDGFSCYNKPIGVNFPHPTR
Bm MIRIYIIVYLFFIIIFIVLKSKRGNDNDNDNDNDNDNDSDHEFSCVNRPIGVHFPHPTK
HaEPV MIICYIIYAIIIICIILLIKSTK.....INQDNHTPDPENEICPPGVFGNIPHPYY
Type 145 Ac CTAYYLCP...HKVQMFCELNHEFDLDSASCKPIVYDHTGSGCTARMYRNLLL*
Bm CTAYYICP...HKVQMFCESNHEFDLDSASCKPIVYDRTGSGCAARMYRNLLL*
Op CNAYYMCP...HKVRMFCDPGHEFELDSATCVPIEYDCLGSGCTARLYRNLLL*
Type 150 Ac CDAFYMCVGLNQKLELICPEGFEFDPDVKNCVPI....SDYGCTANQN*
Bm CNAFYMCVGINHRLELLCSEGFEFDPNVKDCVPI....SDYGCTANQNKNKNKIY*
HaEPV CNRFYLCAAGNAIL.LFCAEGHEYDPTIRTCVLI....SEYGCTANQNRKKSKRK*

Figure 1(c)

		*		*	*		*	*	*	
viral 11K family	X_{25-50}	CX_5	$GX_{5-10}CX_5C$		X_{5-12}			CX_{12}	CX_{5-12}	GCX_{1-15}
defensin family	X_{56}	$CX_{4-8}GX_{6-7}$		CX_3C		$X_3GX_2GGXCX_{4-5}CX$				CX_{1-13}

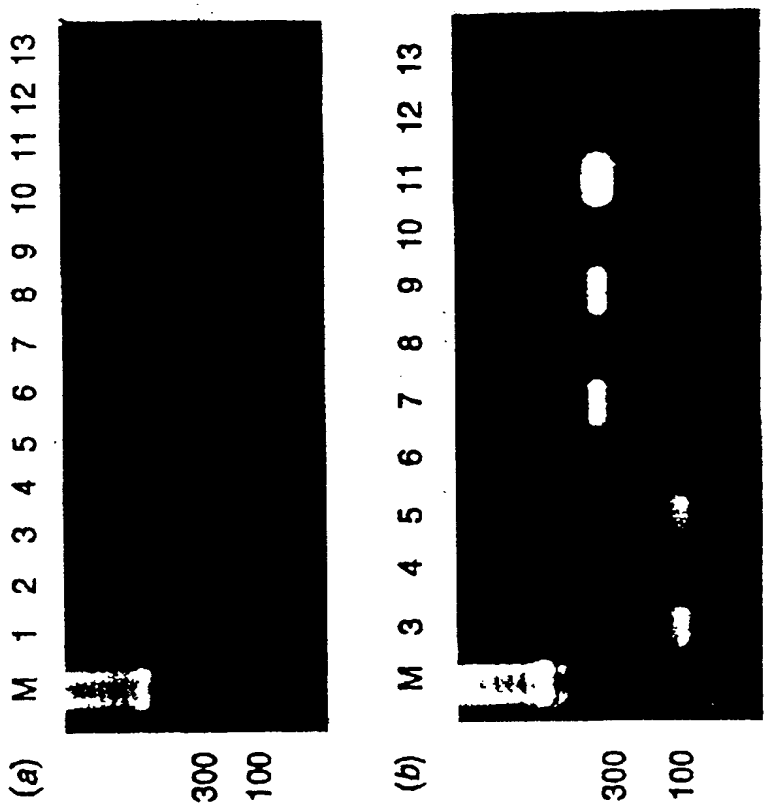


FIGURE 2

FIGURE 2 Cont.

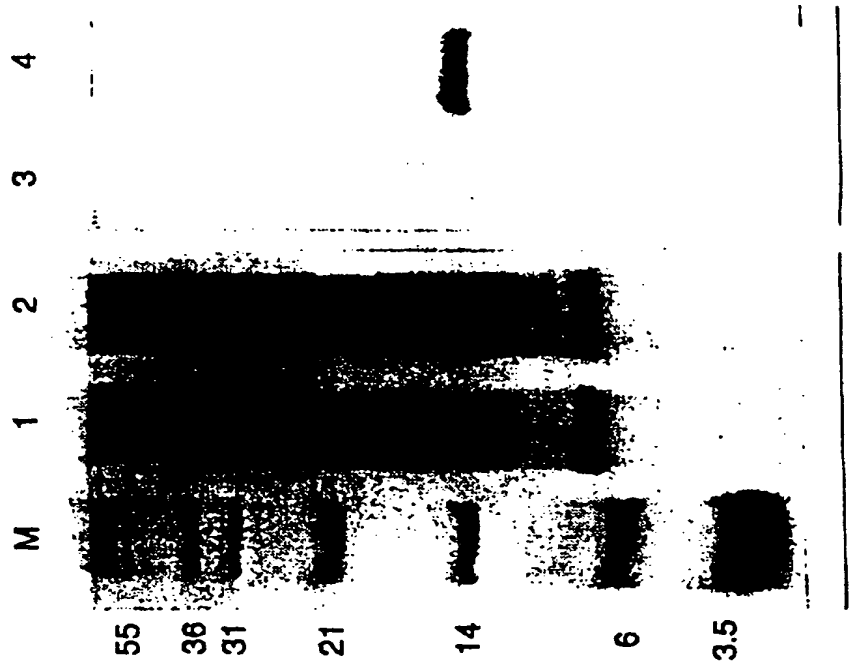


FIGURE 3

Figure 4(a)

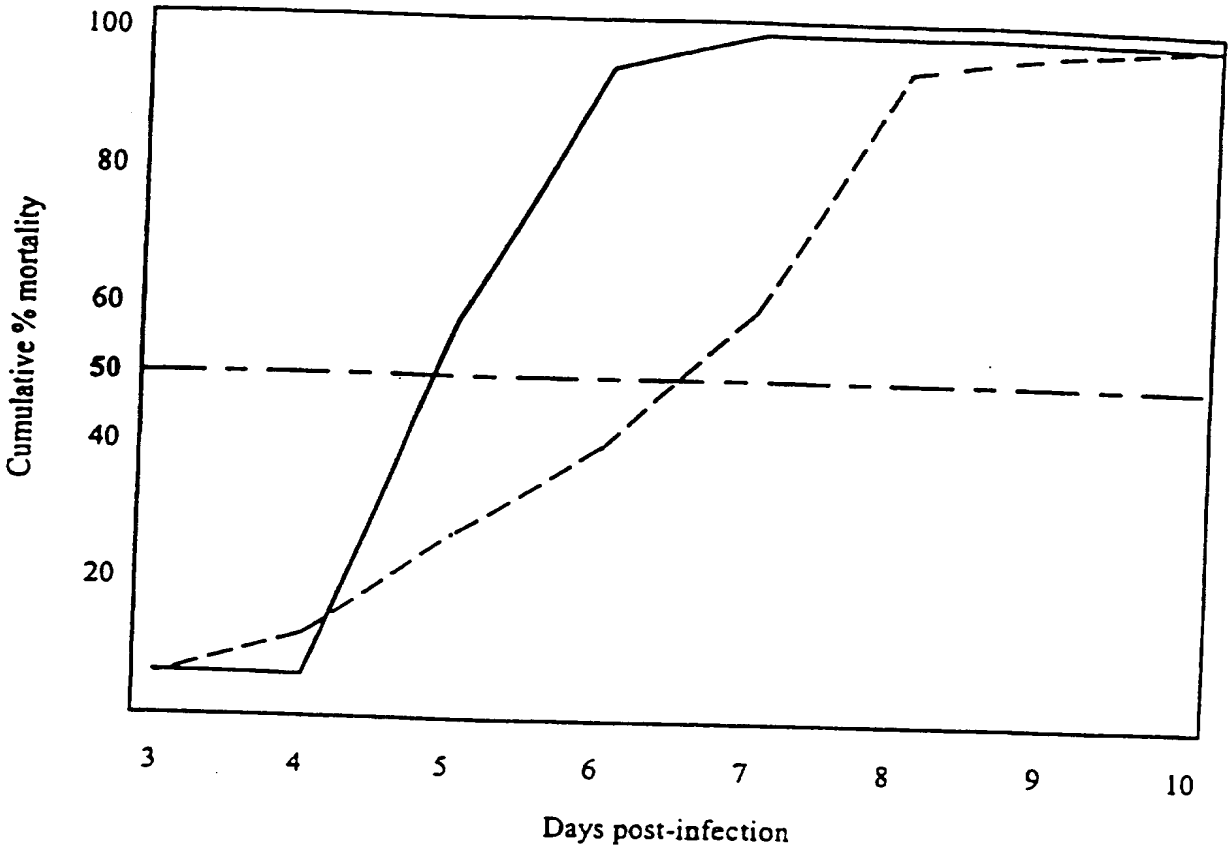
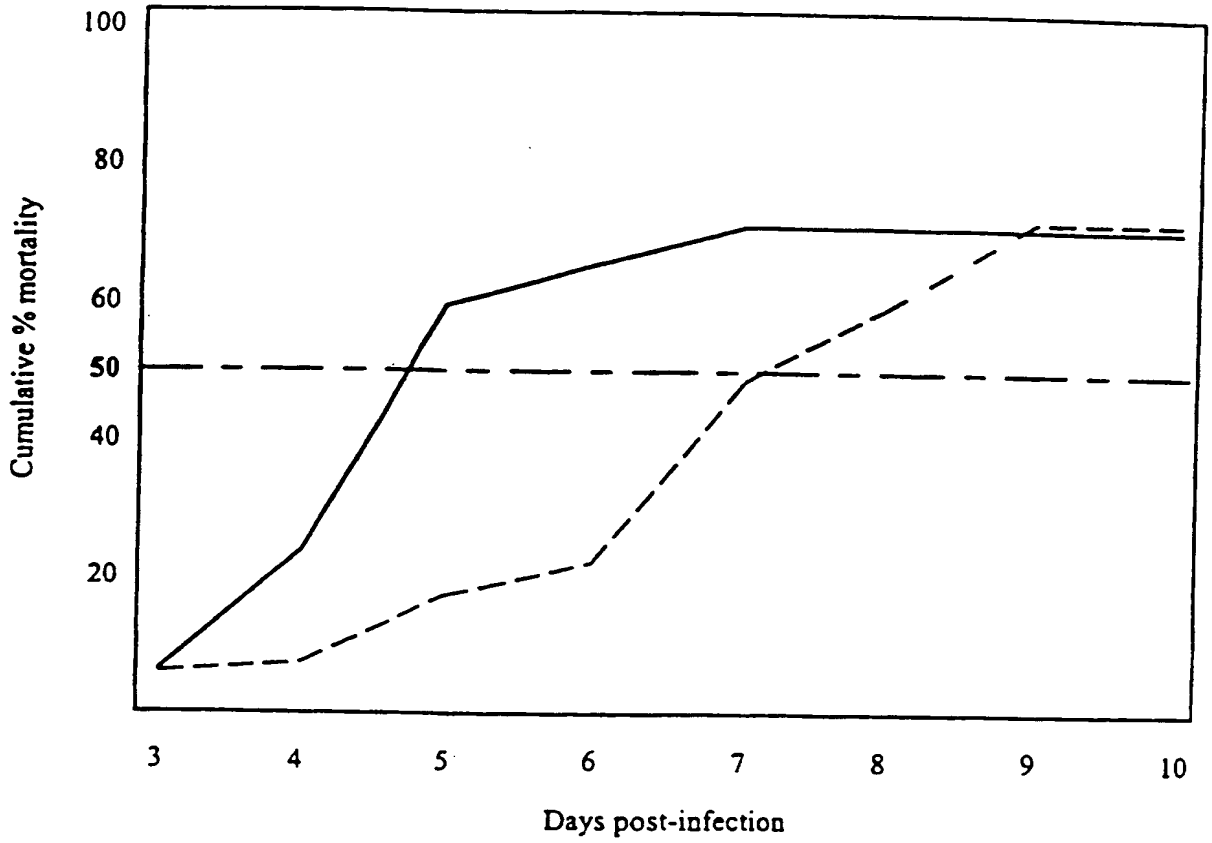

100
80
60
50
40
20
Cumulative % mortality
3
4
5
6
7
8
9
10
Days post-infection

Figure 4(b)


100
80
60
50
40
20
Cumulative % mortality
3
4
5
6
7
8
9
10
Days post-infection

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00615

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C07K 14/065

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K; C12N; A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT: (INSECTICID:()(PROTEIN# OR POLYPEPTIDE#)) AND (ENTOMOPOXVIR: OR NUCLEAR() POLYHEDR: OR OPMNVP OR ACMNVP OR EPV OR HAEPV OR BACULOVIR:)
CA via STN: SEQ - GYFGLNADP OR YGCTANQN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AU,B, 42973/93 (668734)(Commonwealth Scientific and Industrial Research Organisation) 23 December 1993 Example 2, Claims, Figure 1	1-6, 9-11, 14-28
P,X	AU,A, 28972/95 (NATURAL ENVIRONMENT RESEARCH COUNCIL) 18 January 1996 Table 1, SEQ ID No. 1, Claims	1-9, 12-18, 20-22, 24-28
X	Virology, Volume 202, No. 2, 1994, pages 586-605, AYRES M D et al., "The complete DNA sequence of Autographa californica nuclear polyhedrosis virus"	1-9,12-18, 20-22, 24-28

☐ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 December 1996	8 JAN 1997
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer BARRY SPENCER Telephone No.: (06) 283 2284

Form PCT/ISA/210 (second sheet) (July 1992) copbko

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 96/00615

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	28972/95	GB	9413420	WO	9601320		
AU	42973/93	CN	1083527	EP	646172	IL	106038
		JP	8501204	WO	9325666	ZA	9304279

END OF ANNEX